British Journal of Pharmacology (2000) 130, 1809–1816

www.nature.com/bjp

Calcium-pH crosstalks in rat mast cells: cytosolic alkalinization, but not intracellular calcium release, is a sufficient signal for degranulation

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1 The aim of this work was to study the relationship between intracellular alkalinization, calcium fluxes and histamine release in rat mast cells. Intracellular alkalinization was induced by nigericin, a monovalent cation ionophore, and by NH_4Cl (ammonium chloride). Calcium cytosolic and intracellular pH were measured by fluorescence digital imaging using Fura-2-AM and BCECF-AM.

2 In rat mast cells, nigericin and NH_4Cl induce a dose-dependent intracellular alkalinization, a dose-dependent increase in intracellular calcium levels by releasing calcium from intracellular pools, and an activation of capacitative calcium influx.

3 The increase in both intracellular calcium and pH activates exocytosis (histamine release) in the absence of external calcium. Under the same conditions, thapsigargin does not activate exocytosis, the main difference being that thapsigargin does not alkalinize the cytosol.

4 After alkalinization, histamine release is intracellular-calcium dependent. With 2.5 mM EGTA and thapsigargin the cell response decreases by 62%.

5 The cytosolic alkalinization, in addition to the calcium increase it is enough signal to elicit the exocytotic process in rat mast cells.

British Journal of Pharmacology (2000) **130,** 1809–1816

Keywords: Mast cells; calcium; alkalinization; cytosolic pH; fura-2; exocytosis; histamine

Abbreviations: P_3, inositol 1,4,5-trisphosphate; NH_4Cl, ammonium chloride; pHi, intracellular pH; s, seconds; Tg, thapsigargin

Introduction

Rat mast cells are a cell type often used in signal transduction studies because they secrete their granules through a fast exocytosis process, which can be used as a functional model. In this process histamine and other biologically active substances are secreted. Mast cells are mainly involved in pathophysiological episodes such as asthma, allergies, inflammation or intestinal helminth infestations. Also, they seem to have some physiological role, though not well established, in the control of lipid levels, central nervous system blood flow, etc (Marone, 1995). These cells are non-excitable, their low membrane potential being regulated by monovalent plasma-membrane exchange through several exchangers (Bronner *et al*., 1989; Cabado *et al*., 1994; 1998).

Intracellular pH is crucial to different cellular functions. Changes in intracellular pH take place in response to growth, tumoral promoters, secretory processes or changes in membrane conductance or permeability. Na^+/H^+ exchange and different CO_3H^- transporters are the main mechanisms that cells use to control intracellular pH levels (Thomas, 1989). We have recently reported in these cells the existence of both a new Na^+-independent, HCO_3^--dependent alkalinizing mechanism (Vilariño *et al*., 1998; 1999), and a Na^+/Ca^{2+} exchanger (Alfonso *et al*., 1999). Besides, these cells have an important Na^+/H^+ exchanger that recover intracellular pH after an intracellular acidification and it is positively modulated by protein kinase C (PKC) (Alfonso *et al*., 1994a).

The modulation of cellular pH is associated in a complex way to cellular function. Several studies show that the cellular response to a variety of growth and vasoactive factors was associated with a cytosolic alkalinization (Aviv, 1994). In response to receptor stimulation, important changes in the intracellular calcium concentration take place by the release of calcium from inositol 1,4,5-trisphosphate pools (IP_3-pools), and, as a consequence, by the influx of extracellular calcium across the plasma membrane (Putney, 1991). In several cellular models cytosolic alkalinization is a sufficient signal to release calcium from intracellular pools (Dettbarn & Palade 1991; Nitschke *et al*., 1996); however, this alkalinization-induced release of calcium is not always related with a significant calcium entry (Yodozawa *et al*., 1997). Following the increase in cytoplasmic calcium levels after cellular activation, an intracellular acidification may occur in a variety of cell types (Ives & Daniel, 1987; Gertler & Pecht, 1988). In fact, in many cells an active transport system, that exchanges extracellular protons for cytosolic calcium through the plasma membrane Ca^{2+}-ATPase, is activated after the increase in cytosolic calcium levels (Tsukamoto *et al*., 1991; Aviv, 1994; Carafoli & Stauffer, 1994). This increase in cellular activity and the stimulation of the Ca^{2+}-ATPase impose an acid load that promotes the activation of the Na^+/H^+ antiport. Thus, the combined action of the Na^+/H^+ exchange on the plasma membrane and the Ca^{2+}-ATPase in the endoplasmic reticulum, and the Ca^{2+}-membrane ATPase/H^+ exchange serve to regulate intracellular calcium levels.

Cytosolic calcium increase is a necessary step to mast cells exocytosis (Kassel *et al*., 1995), even though the presence of this ion in the extracellular medium is not necessary to trigger histamine release (Botana *et al*., 1992). In these cells, intracellular pH regulation is linked to changes in cytosolic

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calcium and to the inhibition of secretion induced by different stimuli (Lee *et al*., 1992; Jensen *et al*., 1998), bicarbonate playing an important modulatory role which is linked to calcium (Vilariño *et al*., 1999). In previous studies we have reported the functional links of the mechanisms that contribute to regulate acid loads and cell activation in rat mast cells (Botana *et al*., 1992; Alfonso *et al*., 1994a,b; 1998; Vilariño *et al*., 1998), and the relevance of calcium in these cells response (Alfonso *et al*., 1994a; Vilariño *et al*., 1999). In this paper we further study the crosstalks between intracellular alkalinization, cytosolic calcium and histamine release.

Methods

Chemicals

Nigericin and ethilen-glicol-bis(b-aminoethylether) N,N,N′,N′-tetracetic acid (EGTA) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); ammonium chloride and orthophtalaldehyde were from Merck (Darmstadt, Germany); Percoll® was from Pharmacia (Uppsala, Sweden); thapsigargin was from Alexis Corporation (Läufelfingen, Switzerland); 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) and FURA-2 AM were from Molecular Probes (Eugene, OR, U.S.A.).

Mast cell preparation

Mast cells were obtained by lavage of pleural and peritoneal cavities of Sprague-Dawley rats (200–400 g) as described previously (Botana, 1987). The composition of physiological saline solution was (mM): Na^+ 142.3, K^+ 5.94, Ca^{2+} 1, Mg^{2+} 1.2, Cl^- 126.2, HCO_3^- 22.85, PO_4H^{2-} 1.2, SO_4^{2-} 1.2. In all the experiments the incubation medium was equilibrated with CO_2 and the final pH was adjusted to 7.0 prior to use.

The unpurified cellular suspension contained 4–8% mast cells, $1.5-2\times10^6$ per rat. All the experiments were carried out with purified mast cells, except those studying mediator release.

Cell purification

Cells pooled from three rats were purified by centrifugation through 4 ml of isotonic Percoll at $400\times g$ for 10 min. Percoll was eliminated by washing three times with the medium described above at $100\times g$ for 5 min. Cell purity was always higher than 95%. Cell viability was studied by the Trypan blue exclusion test and was always higher than 97%.

Cell incubation

Twenty-five microlitres of a freshly prepared concentrated solution of NH_4Cl were added to the incubation medium to attain a final volume of 0.925 ml and preincubated. When the medium reached 37°C, 25 ml of a cell suspension containing $1-1.5\times10^5$ mast cells, were added to each tube. Incubations were carried out in a bath at 37°C for 10 min.

The incubations were stopped by immersing the tubes in a cold bath. After centrifugation at $1000\times g$ max for 3 min, the supernatants were collected and decanted into other tubes for histamine determination. Appropriate controls to determine spontaneous histamine release in the absence of stimuli were executed in each experiment.

Histamine release assay

Histamine was assayed spectrofluorimetrically both in pellet (residual histamine) and supernatants (histamine released) by Shore's method (Shore, 1971) in a spectrofluorometer Kontron SFM 25. However, 0.1% orthophthalaldehyde was employed. Trichloroacetic acid was added (7%, final concentration) to prevent reaction because proteins interfere with histamine release assay. To ensure total histamine, pellets were sonicated for 60 s in 0.8 ml of 0.1 N HCl. In NH_4Cl experiments histamine released was determined only in pellet because this chemical interferes with histamine fluorescence. Results are expressed as a percentage of histamine released with respect to total histamine content.

Measurement of cytosolic free calcium and intracellular pH image processing

Purified rat mast cells were loaded with Fura-2 AM (0.3 μM) and with 0.1 μM BCECF-AM for 10 min at 37°C. Loaded cells were washed three times ($400\times g$/2 min) and allowed to attach to poly-L-lysine-coated 22-mm glass coverslips for 10 min. The glass coverslips were inserted into a thermostated chamber (Life Science Resources, U.K.) and cells were viewed with a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40×-immersion UV-Fluor objective). The chamber was used in the open bath configuration and additions made by aspiration and addition of fresh bathing solution. Intracellular calcium concentration and pH were obtained from the images collected by quadruple excitation fluorescence with a Life Science Resources (U.K.) equipment. The light source was a 175 W xenon lamp, and light reached the objective with optic fibre. The excitation wavelengths for Fura were 340 and 380 nm, with emission at 505 nm, and for BCECF 440 and 490 nm, both for excitation, and 530 nm for emission. The calibration of the fluorescence *vs* intracellular calcium was made by using the method of Grynkiewicz (Grynkiewicz *et al*., 1985). The calibration of fluorescence *vs* pHi was made using nigericin in K^+ solution as per Thomas *et al*. (1979). Briefly, a calibration curve was obtained with four known values of pH, measuring the fluorescence ratio obtained in the presence of nigericin for each pH value. With these values we obtained in

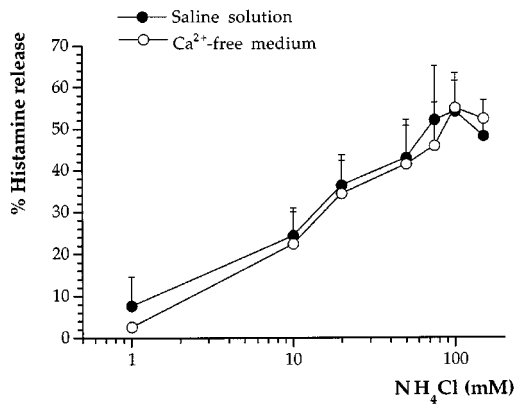

Figure 1 Dose-response of histamine released in rat mast cells in the presence of NH_4Cl. Different concentrations of NH_4Cl were added and histamine release was checked after 10 min of incubation of rat mast cells in medium with (closed circles) and without calcium (open circles). Mean ± s.e.mean of six experiments.

each experiment a calibration curve (ratio *vs* pH) which was used to transform any ratio value to pH.

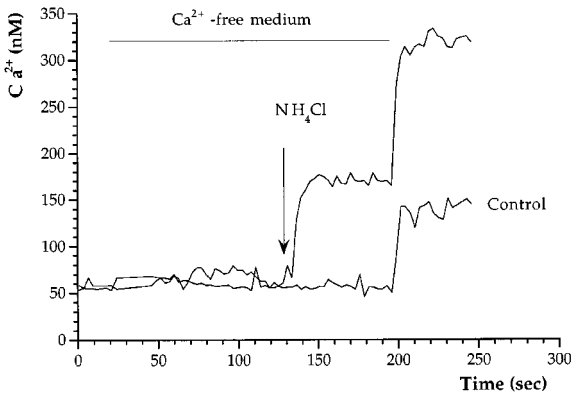

Figure 2 Effect of NH_4Cl in cytosolic calcium levels in rat mast cells. Ca^{2+} was removed from the extracellular medium to check intracellular pools contribution to calcium increase induced by NH_4Cl. The arrow indicates the addition of 20 mM NH_4Cl. 1 mM Ca^{2+} is added again after calcium release from the pools to restore external calcium conditions. Mean of 60 cells from a single experiment. Similar experiments were carried out four more times.

Statistical analysis

Results were analysed using the Student's *t*-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± s.e.mean.

Results

In order to know the importance of cytoplasmic alkalinization in histamine release we checked the effect of ammonium chloride (NH_4Cl) in rat mast cells exocytosis. This chemical is known to produce intracellular alkalinization (Roos & Boron, 1981). As Figure 1 shows, NH_4Cl induced a maximum 60% dose-dependent histamine release. This release is not dependent on the presence of extracellular calcium. Under these conditions, which are enough to induce the cellular exocytosis, we checked the changes on cytosolic calcium, as shown in Figure 2. The results obtained indicate that after the addition of 20 mM NH_4Cl (which is the EC_{50}) in a Ca^{2+}-free medium, a fast release of calcium from intracellular pools takes place, while control cells show no calcium increase. Accordingly, and after the addition of calcium, a significant capacitative calcium influx was observed, the increase of calcium being higher in the

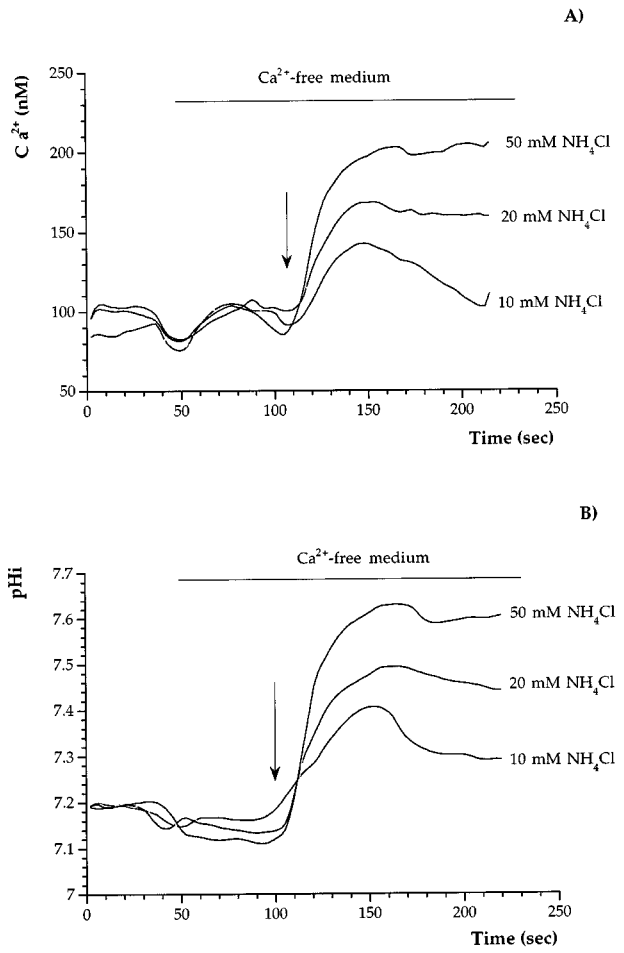

Figure 3 Effect of different concentrations of NH_4Cl in cytosolic calcium and intracellular pH levels in rat mast cells. (A) Variation of cytosolic Ca^{2+} levels in the presence of 10, 20 and 50 mM NH_4Cl in a calcium free medium. (B) Intracellular pH profile in cells subject to the protocol in (A) in a calcium free medium. Arrows indicate the addition of NH_4Cl. Mean of three experiments by duplication (approximately 40 cells/single experiment).

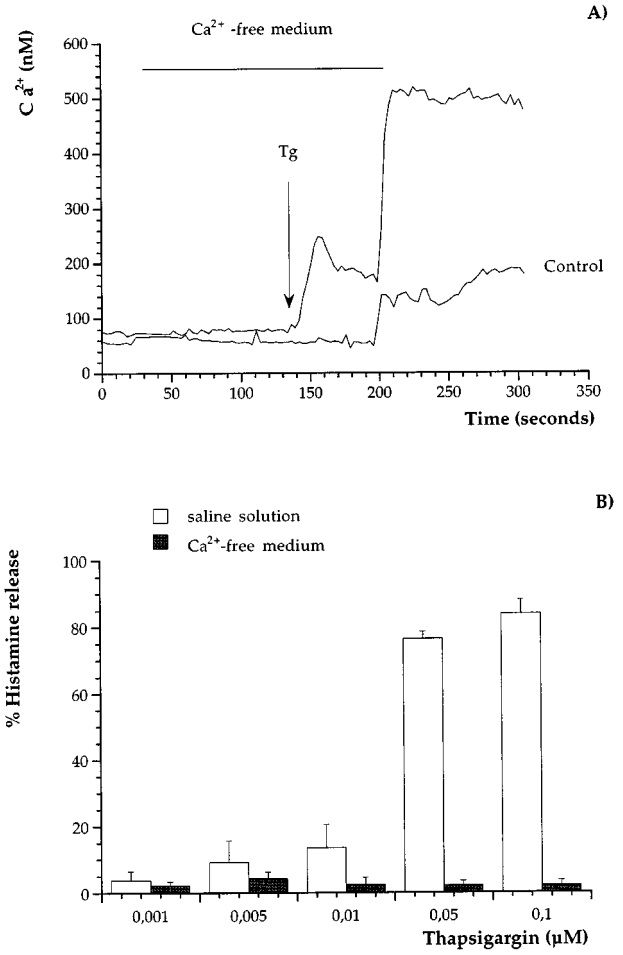

Figure 4 (A) Effect of thapsigargin on cytosolic calcium levels in rat mast cells. The calcium was present when indicated. The arrow indicates the addition of 1 μM thapsigargin. Mean of 80 cells. (B) Histamine release in rat mast cells in the presence of thapsigargin. Different concentrations of thapsigargin were added and histamine release was checked after 10 min of incubation in medium with and without calcium. Mean ± s.e.mean of four experiments.

presence of NH_4Cl. Figure 3 shows that the concentrations that induced a response around 50% maximum histamine release (10, 20 and 50 mM NH_4Cl), caused both an intracellular alkalinization and a cytosolic calcium increase in a similar dose-dependent fashion. Figure 3A shows the gradual cytosolic calcium increase, 140, 170 and 210 nM, after the addition of 10, 20 and 50 mM NH_4Cl, respectively, in a Ca^{2+}-free medium. In these conditions, as Figure 3B shows, intracellular pH increases from 7.2 to 7.4, 7.5 and 7.64 with NH_4Cl concentration, and after 5 min the pH returns to initial resting values.

The response elicited by NH_4Cl indicates an increase in calcium levels in the absence of external calcium. Therefore, we decided to compare this effect in cells treated with thapsigargin. Thapsigargin is a tumour-promoting sesquiterpene lactone that inhibits Ca^{2+}-ATPase from intracellular pools (Thastrup *et al*., 1987). As a consequence, thapsigargin induces calcium pool emptying. As shown in Figure 4A, thapsigargin does increase, in the absence of external calcium, cytosolic calcium levels and capacitative calcium influx. The pattern does not relate to cellular exocytosis, since in the absence of external calcium there is no release of histamine, as shown in Figure 4B. This makes a critical difference with regards to NH_4Cl.

So far, results show that NH_4Cl elicits histamine release, cytosolic alkalinization and an increase in cytosolic calcium levels in the absence of external calcium. On the other hand, in the same conditions thapsigargin does not induce histamine release even if it induces increases in cytosolic calcium. Therefore to clarify the differences between NH_4Cl and thapsigargin, we studied whether or not NH_4Cl and thapsigargin release calcium from the same pools, and we studied the change in cytosolic pH induced by thapsigargin. As Figure 5A shows, NH_4Cl induces a significant calcium release from intracellular pools. The subsequent thapsigargin addition only induces more calcium release at low NH_4Cl concentrations, indicating that thapsigargin pools are already depleted by the higher concentrations of NH_4Cl. This observation also suggests that both compounds affect the same pools. On the other hand, intracellular pH profiles were not modified by thapsigargin under these conditions, as shown in Figure 5B.

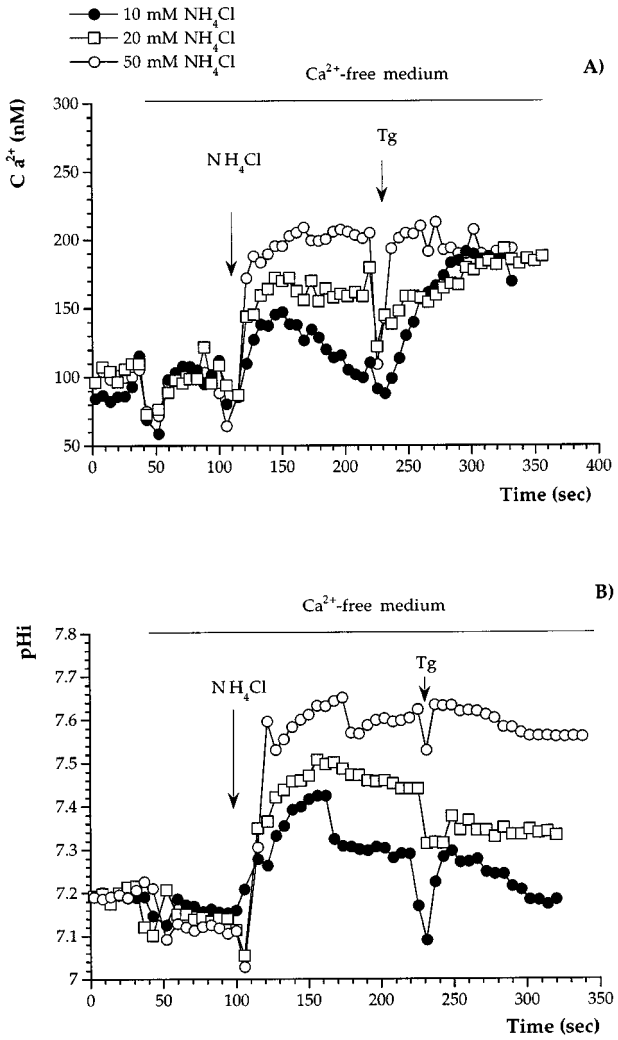

Figure 5 Effect of NH_4Cl and thapsigargin on cytosolic calcium concentration and on intracellular pH profile in rat in rat mast cells. (A) Cytosolic calcium profile from cells stimulated with incremental NH_4Cl concentrations (10, 20 or 50 mM) first and after 2 min with 1 μM thapsigargin in a calcium free medium. (B) Intracellular pH profile for rat mast cells subject to the protocol in (A). Arrows indicate the addition of NH_4Cl and thapsigargin. Mean of three experiments by duplicate (approximately 40 cells/single experiment).

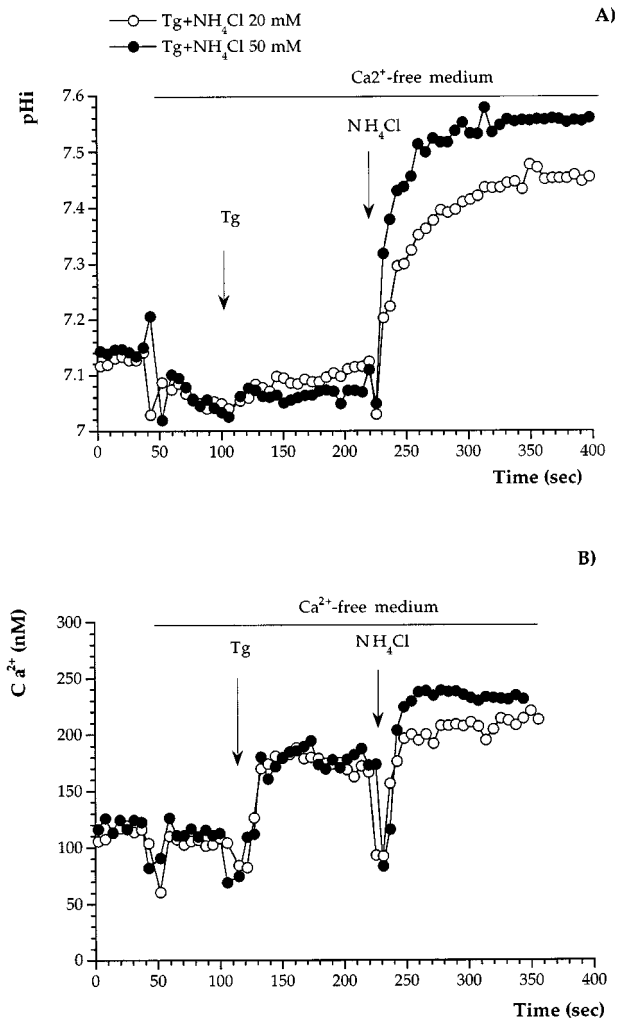

Figure 6 Effect of thapsigargin and NH_4Cl on intracellular pH profile and on cytosolic calcium concentration in rat in rat mast cells. (A) Intracellular pH profile from cells stimulated with 1 μM thapsigargin first and after 2 min with incremental NH_4Cl concentrations (20 or 50 mM) in a calcium free medium. (B) Cytosolic calcium profile for rat mast cells subject to the protocol in (A). Arrows indicates the addition of thapsigargin and NH_4Cl. Mean of three experiments by duplicate (approximately 40 cells/single experiment).

Figure 6A shows that thapsigargin neither alkalinizes pHi in resting conditions nor prevents NH_4Cl alkalinization. However, Figure 6B shows that after emptying intracellular pools with thapsigargin, the addition of high concentrations of NH_4Cl induces an additional calcium release from intracellular stores. Then we checked the release of histamine induced by NH_4Cl after emptying intracellular pools with thapsigargin. As Figure 7 shows, we did not observe any modifications on histamine release induced by NH_4Cl. However when calcium released from intracellular pools was eliminated by the addition of EGTA (a calcium chelator), histamine release induced by NH_4Cl was inhibited by 60% (shown in Figure 7B). In addition, and completing results of Figure 4, this graphic shows that even at concentrations of thapsigargin used to fully empty intracellular pools (2 μM thapsigargin) in a calcium free medium, there is no histamine release.

In order to clarify the role of alkalinization on histamine release, we checked the effect of nigericin, which is an ionophore selective to monovalent cations, particularly H^+ and K^+ (Thomas *et al*., 1979). Usually, intracellular pH is in any cellular model about 0.1–0.2 units more acidic than extracellular pH. In the presence of nigericin, both intracellular and extracellular pH become equal; hence intracellular pH will be artificially increased (more alkaline). Figure 8A shows the increase in 0.15 pH units of intracellular pH that takes place after the addition of nigericin. Figure 8B shows the increase in intracellular calcium induced by nigericin. In these conditions, nigericin induces a dose-dependent histamine release, as Figure

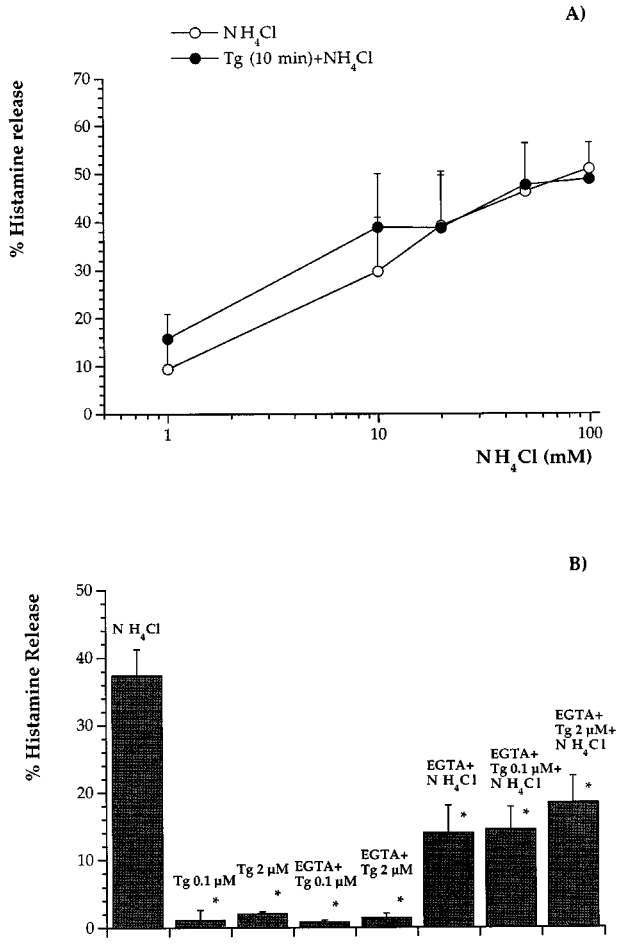

Figure 7 (A) Dose-response of histamine release in rat mast cells stimulated with NH_4Cl after a 10 min preincubation with 1 μM thapsigargin in a calcium-free medium (closed circles). Control of NH_4Cl (open circles). (B) Percentage of histamine release in rat mast cells stimulated with 20 mM NH_4Cl for 10 min after 20 min preincubation with: 2.5 mM EGTA, 0.1 μM thapsigargin and 2.5 mM EGTA, or 2 μM thapsigargin and 2.5 mM EGTA, in a calcium-free medium. Significant differences with respect to the 20 mM NH_4Cl-control are indicated with an asterisk. Mean ± s.e.mean of six experiments.

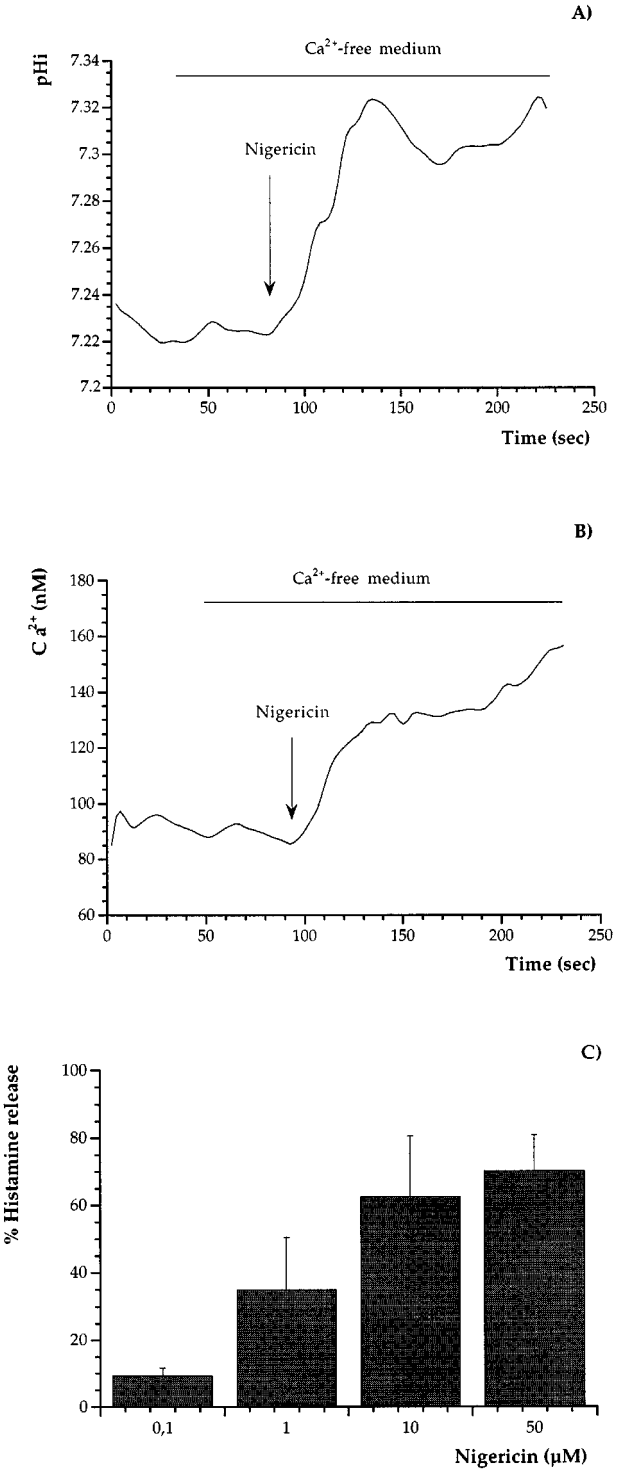

Figure 8 Effect of nigericin on intracellular pH profile, on cytosolic calcium concentration and on histamine release in rat mast cells. (A) Intracellular pH profile from cells stimulated with 0.1 μM nigericin in a calcium free medium. (B) Cytosolic calcium profile for rat mast cells subject to the protocol in (A). Arrows indicate the addition of nigericin. Mean of three experiments by duplicate (approximately 40 cells/single experiment). (C) Dose-response of histamine release in rat mast cells stimulated with nigericin for 10 min in a calcium-free medium. Mean ± s.e.mean of six experiments.

8C shows, with a maximum of 70% released in a calcium-free medium. These results suggest again that a cytosolic alkalinization induces histamine release.

Discussion

This paper demonstrates that cytosolic alkalinization is a sufficient signal to release histamine in rat mast cells, and as far as we know, this is a new observation.

Evidences indicating the relationship between intracellular calcium and pHi have been described in different cellular models (Siffert *et al*., 1990a,b; Alfonso *et al*., 1994a,b). Cytosolic alkalinization is related to calcium release from intracellular pools (Dettbarn & Palade, 1991; Taylor *et al*., 1992; Nitschke *et al*., 1996; Yodozawa *et al*., 1997; Vilariño *et al*., 1999), and cytosolic acidification is related to calcium extrusion (Ives & Daniel, 1987; Gertler & Pecht, 1988) and calcium mobilization (Berk *et al*., 1987). In rat lacrimal and pancreatic acinar cells (Yodozawa *et al*., 1997; Speake & Elliott, 1998), and as we show in this paper in rat mast cells, an intracellular alkalinization induced by nigericin or NH_4Cl increases cytosolic calcium levels and calcium release from intracellular pools. The mechanisms of both nigericin and NH_4Cl are different, the first is an ionophore and the second increases the concentration of intracellular alkali. But regardless of the different mechanisms, the results are the same, which suggest the effect is related to internal pHi, and not to the mechanisms of alkalinization. Therefore, we can conclude that an intracellular alkalinization is associated with an increase in cytosolic calcium levels in rat mast cells, as described in other cellular models (Ekokoski & Törnguist, 1994; Ilino *et al*., 1994). The cytosolic calcium increase is clearly a net result of a balance between extracellular calcium uptake and calcium extrusion from intracellular calcium pools. The results obtained in the presence of NH_4Cl confirm this conclusion, since the alkalinization induced by NH_4Cl is enough to empty calcium pools and activate capacitative calcium influx.

In RBL-2H3 cells, an acidification is associated with an inhibition of secretion induced by both antigen and ionomycin (Lee *et al*., 1992). In MC9 mast cells, histamine release induced by antigen was inhibited after the alkalinization induced by antigen was blocked (Fanous & Garay, 1993). On the other hand, the secretion induced by compound 48/80 in rat mast cells increases when intracellular pH becomes more alkaline (Jensen *et al*., 1998). Therefore, experimental evidence has shown an increase or a decrease in histamine release depending on cytosolic pH. Our observation, regardless of the presence of external calcium, that a cytosolic alkalinization induces histamine release, suggests a clear dependence on internal calcium, assuming exocytosis does not exist without an increase of cytosolic calcium, which our data obtained in the presence of EGTA further confirms. Therefore, we might speculate that in the presence of NH_4Cl, after an intracellular alkalinization, calcium release from intracellular pools could be enough signal to induce histamine release. This mechanism is very interesting, since we have demonstrated in a previous work that only in the presence of extracellular calcium does thapsigargin induce histamine release in rat mast cells (Alfonso *et al*., 1994b). This drug induces calcium release from intracellular pools, the same stores than NH_4Cl, but no effect was observed on intracellular pH levels. A similar suggestion, though by different approaches, was reported earlier in basophils to prove that the initial transient elevation associated with the mobilization of intracellular calcium is an insufficient signal for degranulation (MacGlashan & Botana, 1993). Therefore, we can conclude that, at least in the case of mast cells, the results suggest that cytosolic alkalinization, but not intracellular calcium release, is a sufficient signal for degranulation.

Our results in rat mast cells confirm that alkalinization releases calcium from thapsigargin-dependent stores. Several theories explain this release in other cellular models, such as an increase in IP_3 levels or IP_3-receptor sensitivity after intracellular alkalinization (Nitschke *et al*., 1996; Yodozawa *et al*., 1997; Speake & Elliott, 1998), an increased sensitivity of calcium release channels (Dettbarn & Palade, 1991), or maybe an unknown mechanism (Nitschke *et al*., 1996). The small calcium release observed after high NH_4Cl concentrations in thapsigargin-depleted cells might indicate either a residual release from the same pools that thapsigargin cannot fully deplete (Steenbergen & Fay, 1996). We can consider also the contribution of other nonthapsigargin sensible calcium pool (Gill *et al*., 1996) releasable by NH_4Cl, which suggests the functional heterogeneity of the Ca^{2+} mobilization from stores (Hirose & Iino, 1994).

It is noteworthy pointing out that histamine release obtained in the presence of NH_4Cl was reproduced in the presence of nigericin. This is important to disregard any leakage of histamine by a blockade of uptake and retention of biogenic amines by mast cells granules in the presence of ammonium (Ludowyke & Lagunoff, 1986). Moreover, histamine release observed in the presence of NH_4Cl behaves in a dose-response fashion, and also matches the increase in intracellular pH observed. In addition, the alkalinization-induced histamine release in a calcium-free medium can be modulated by different intracellular pathways, which eliminates any unspecific leakage (manuscript in preparation).

This key role of alkalinization, as first inductor of histamine release, is, as far as we know, the first time this has been clearly proposed for mast cells. It has been earlier described an intracellular alkalinization associated to histamine release induced by compound 48/80 (Friis & Johansen, 1996; Jensen *et al*., 1998). This alkalinization seems to be mediated through the Na^+-H^+-exchanger stimulation, and reproduced by phorbol esther and ionomycin. It seems to play an important rather than essential role for histamine release, because even when the alkalinization was blocked, compound 48/80 did induce histamine release (Friis & Johansen, 1996). In the present paper we demonstrate that the alkalinization has a primordial role for histamine release, because the release of calcium from intracellular pools is not enough to release histamine. Clearly, further studies are needed to understand the mechanism that induces calcium-depleted pools after alkalinization, and to determine which effector system is stimulated to release histamine. As happens with many secretory stimuli, the increase in cytosolic pH levels triggers several interrelated signalling pathways which presumably mediate secretion (Shefler *et al*., 1998; 1999); this matter requires deeper studies to fully understand the mechanism. It is also important to address in the future the importance of pHi recovery after NH_4Cl incubation, and the influence in histamine release of the various transport systems present on the cells (Alfonso *et al*., 1998; 1999), In most cells, intracellular pHi is restored to resting values after an alkali load by extrusion of intracellular HCO_3^- in exchange for extracellular C^- through the Na^+-independent $C^-HCO_3^-$ exchanger (Boron & Knakal, 1989). Recent data reports that in rat mast cells (Vilariño *et al*., 1998) there is a mechanism partially independent of exchangers that modulates H^+ fluxes and is closely regulated by Ca^{2+} and PKC. This regulation is further

potentiated in a cellular calcium-dependent response in HCO_3^--free conditions (Vilariño *et al*., 1999).

Conclusion

In a calcium-free medium only the combination of cytosolic alkalinization and calcium increase, as for NH_4Cl or nigericin, induces histamine release in rat mast cells. The first (cytosolic alkalinization), but not the second (calcium increase), is a sufficient signal to trigger the exocytosis.

This work was funded with grants FEDER-CICYT-1FD97-0153 and Xunta Galicia PGIDT99INN26101.

References

ALFONSO, A., BOTANA, M.A., VIEYTES, M.R. & BOTANA, L.M. (1994a). Functional characterization of the Na^+-H^+ exchanger in rat mast cells: crosstalks between different kinase pathways. *Eur. J. Pharmacol.,* **267,** 289–296.

ALFONSO, A., BOTANA, M.A., VIEYTES, M.R. & BOTANA, L.M. (1998). Sodium, PMA and calcium play an important role on intracellular pH modulation in rat mast cells. *Cell. Physiol. Biochem.,* **8,** 314–327.

ALFONSO, A., BOTANA, M.A., VIEYTES, M.R., LOUZAO, M.C. & BOTANA, L.M. (1994b). Effect of signal transduction pathways on the action of thapsigargin on rat mast cells. Crosstalks between cellular signalling and cytosolic pH. *Biochem. Pharmacol.,* **47,** 1813–1820.

ALFONSO, A., LAGO, J., BOTANA, M.A., VIEYTES, M.R. & BOTANA, L.M. (1999). Characterization of the $Na^+/Ca2^+$ exchanger on rat mast cells–Evidence for a functional role on the regulation of the cellular response. *Cell. Physiol. Biochem.,* **9,** 53–71.

AVIV, A. (1994). Cytosolic $Ca2^+$, Na^+/H^+ antiport, protein kinase C trio in essential hypertension. *Am. J. Hypertens,* **7,** 205–212.

BERK, B.C., BROCK, T.A., GIMBRONE, M.A. & ALEXANDER, R.W. (1987). Early agonist-mediated ionic events in cultured vascular smooth muscle cells. Calcium mobilization is associated with intracellular acidification. *J. Biol. Chem.,* **262,** 5065–5072.

BORON, W.F. & KNAKAL, R.C. (1989). Intracellular pH-regulating mechanism of the squid axon. Interaction between DNDS and extracellular Na^+ and $HCO3^-$. *J. Gen. Physiol.,* **93,** 123–150.

BOTANA, L.M. (1987). Adrenergic agonists do not compete with the antagonist (-)3-[125l]iodocyanopindolol for binding to rat pleural or peritoneal mast cell adrenergic receptor. *Gen. Pharmacol.,* **18,** 263–267.

BOTANA, L.M., ALFONSO, A., BOTANA, M.A., VIEYTES, M.R., LOUZAO, M.C. & CABADO, A.G. (1992). Influence of protein kinase C, cAMP and phosphatase activity on histamine release produced by compound 48/80 and sodium fluoride on rat mast cells. *Agents Actions,* **37,** 1–7.

BRONNER, C., MOUSLI, M., ELENO, N. & LANDRY, Y. (1989). Resting plasma membrane potential of rat peritoneal mast cells is set predominantly by the sodium pump. *FEBS Lett,* **255,** 401–404.

CABADO, A.G., VIEYTES, M.R. & BOTANA, L.M. (1994). Effect of ion composition on the changes in membrane potential induced with several stimuli in rat mast cells. *J. Cell. Physiol.,* **158,** 309–316.

CABADO, A.G., VIEYTES, M.R. & BOTANA, L.M. (1998). Inhibition of Na^+/K^+ ATPase under hypertonic conditions in rat mast cells. *Life Sci,* **63,** 1227–1237.

CARAFOLI, E. & STAUFFER, T. (1994). The plasma membrane calcium pump: functional domains, regulation of the activity and tissue specificity of isoform expression. *Neurobiology,* **25,** 312–324.

DETTBARN, C. & PALADE, P. (1991). Effects of alkaline pH on sarcoplasmic reticulum Ca^{2+} release and Ca^{2+} uptake. *J. Biol. Chem,* **266,** 8993–9001.

EKOKOSKI, E. & TÖRNQUIST, K. (1994). Effects of 5,8,11,14-eicosatetraynoic acid on thapsigargin-induced calcium entry, and intracellular pH in thyroid FRTL-5 cells. *Biochim. Biophys. Acta Mol. Cell. Res.,* **1223,** 274–278.

FANOUS, K. & GARAY, R.P. (1993). Azelastine and allergen transduction signal in MC9 mast cells. *Naunyn Schmiedebergs Arch Pharmacol,* **348,** 515–519.

FRIIS, U.G. & JOHANSEN, T. (1996). Dual regulation of the Na^+/H^+-exchange in rat peritoneal mast cells: Role of protein kinase C and calcium on pHi regulation and histamine release. *Br. J. Pharmacol.,* **118,** 1327–1334.

GERTLER, R. & PECHT, I. (1988). Ionic signalling in mast cells; antigen and ionophore induced changes in cytosolic pH. *Mol. Immunol.,* **25,** 1087–1092.

GILL, D.L., WALDRON, R.T., RYS-SIKORA, K.E., UFRET-VINCENTY, C.A., GRABER, M.N., FAVRE, C.J. & ALFONSO, A. (1996). Calcium pools, calcium entry, and cell growth. *Biosci. Rep.,* **16,** 139–157.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{++} indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440–3450.

HIROSE, K. & IINO, M. (1994). Heterogeneity of channel density in inositol-1,4,5-triphosphate-sensitive Ca^{2+} stores. *Nature,* **372,** 791–794.

ILINO, S., HAYASHI, H., SAITO, H., TOKUNO, H. & TOMITA, T. (1994). Effects of intracellular pH on calcium currents and intracellular calcium ions in the smooth muscle of rabbit portail vein. *Experimental Physiology,* **79,** 669–680.

IVES, H.E. & DANIEL, T.O. (1987). Interrelationship between growth factor-induced pH changes and intracellular Ca^{2+}. *Proc. Natl. Acad. Sci. U.S.A.,* **84,** 1950–1954.

JENSEN, T.B., FRIIS, U.G. & JOHANSEN, T. (1998). Role of physiological HCO3- buffer on intracellular pH and histamine release in rat peritoneal mast cells. *Pflügers. Arch.,* **436,** 357–364.

KASSEL, O., AMRANI, Y., LANDRY, Y. & BRONNER, C. (1995). Mast cell activation involves plasma membrane potential- and thapsigargin-sensitive intracellular calcium pools. *Fundam. Clin. Pharmacol.,* **9,** 531–539.

LEE, R.J., OLIVER, J.M., DEANIN, G.G., TROUP, C.D. & STUMP, R.F. (1992). Importance of bicarbonate ion for intracellular pH regulation in antigen- and ionomycin-stimulated RBL-2H3 mast cells. *Cytometry,* **13,** 127–136.

LUDOWYKE, R.I. & LAGUNOFF, D. (1986). Amine uptake into intact mast cell granules in vitro. *Biochemistry,* **25,** 6287–6293.

MACGLASHAN, D.J. & BOTANA, L.M. (1993). Biphasic Ca^{2+} responses in human basophils. Evidence that the initial transient elevation associated with the mobilization of intracellular calcium is an insufficient signal for degranulation. *J. Immunol.,* **150,** 980–991.

MARONE, G. (1995). Human Basophils and mast cells: biological aspects, ed. Marone, G. In: *Chemical Immunol.* Basel: Karger.

NITSCHKE, R., RIEDEL, A., RICKEN, S., LEIPZIGER, J., BENNING, N., FISCHER, K.G. & GREGER, R. (1996). The effect of intracellular pH on cytosolic Ca^{2+} in HT29 cells. *Pflügers Arch,* **433,** 98–108.

PUTNEY, JR, J.W. (1991). The capacitative model for receptor-activated calcium entry. *Adv. Pharmacol.,* **22,** 251–269.

ROOS, A. & BORON, W.J. (1981). Intracellular pH. *Phys. Rev.,* **61,** 296–434.

SHEFLER, I., TAUBE, Z., MEDALIA, O. & SAGI-EISENBERG, R. (1998). Basic secretagogues activate protein tyrosine phosphorylation and release of arachidonic acid in mast cells via a novel protein kinase C and phosphatidylinositol 3-kinase-dependent mechanism. *Eur. J. Immunol.,* **28,** 3468–3478.

SHEFLER, J., SEGER, R. & SAGI-EISENBERG, R. (1999). Gi-mediated activation of mitogen-activated protein kinase (MAPK) pathway by receptor mimetic basic secretagogues of connective tissue-type mast cells: bifurcation of arachidonic acid-induced release upstream of MAPK. *J. Pharmacol. Exp. Ther.,* **289,** 1654–1661.

SHORE, P.A. (1971). Fluorometric assay of histamine. *Meth. Enz.,* **XVII,** 842–845.

SIFFERT, W., JAKOBS, K.H. & AKKERMAN, J.W. (1990a). Sodium fluoride prevents receptor- and protein kinase C-mediated activation of the human platelet Na^+/H^+ exchanger without inhibiting its basic pHi-regulating activity. *J. Biol. Chem.,* **265,** 15441 – 15448.

SIFFERT, W., SIFFERT, G., SCHEID, P. & AKKERMAN, J.W. (1990b). Na^+/H^+ exchange modulates Ca^{2+} mobilization in human platelets stimulated by ADP and the thromboxane mimetic U 46619. *J Biol. Chem.,* **265,** 719 – 725.

SPEAKE, T. & ELLIOTT, A.C. (1998). Modulation of calcium signals by intracellular pH in isolated rat pancreatic acinar cells. *J. Physiol. (Lond.),* **506,** 415 – 430.

STEENBERGEN, J.M. & FAY, F.S. (1996). The quantal nature of calcium release to caffeine in single smooth muscle cells results from activation of the sarcoplasmic reticulum Ca^{2+}-ATpase. *J. Biol. Chem.,* **271,** 1821 – 1824.

TAYLOR, J.A., BUSCHBAUM, R.N., ZIMNIAK, A. & RACKER, E. (1992). Inositol triphosphate receptors and intracellular calcium. In: *Intracellular Messengers*. pp. 79 – 128. Totowa, New Jersey.

THASTRUP, O., LINNEBJERG, H., BJERRUM, P.J., KNUDSEN, J.B. & CHRISTENSEN, S.B. (1987). The inflammatory and tumor-promoting sesquiterpene lactone, thapsigargin, activates platelets by selective mobilization of calcium as shown by protein phosphorylations. *Biochim. Biophys. Acta.,* **927,** 65 – 73.

THOMAS, J.A., BUCHSBAUM, R.N., ZIMNIAK, A. & RACKER, E. (1979). Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry,* **18,** 2210 – 2218.

THOMAS, R.C. (1989). Cell growth factors: bicarbonate and pHi response. *Nature,* **337,** 601.

TSUKAMOTO, Y., SIGIMURA, K. & SUKI, W.N. (1991). Role of Ca^{+2}/H^+ antiporter in the kidney. *Kidney Int.,* **40,** S90-S94.

VILARIÑO, N., VIEYTES, M.R., VIEITES, J.M. & BOTANA, L.M. (1998). Role of HCO3- ions in cytosolic pH regulation in rat mast cells. Evidence for a new Na^+-independent, HCO3– dependent alkalinizing mechanism. *Biochem. Biophys. Res. Comm.,* **253,** 320 – 324.

VILARIÑO, N., VIEYTES, M.R., VIEITES, J.M. & BOTANA, L.M. (1999). Modulatory effect of HCO3- on rat mast cell exocytosis: Cross-talks between bicarbonate and calcium. *Biochem, Biophys. Res. Commun.,* **260,** 71 – 79.

YODOZAWA, S., SPEAKE, T. & ELLIOTT, A. (1997). Intracellular alkalinization mobilizes calcium from agonist-sensitive pools in rat lacrimal acinar cells. *J. Physiol-Lond.,* **499,** 601 – 611.

(Received January 18, 2000
Revised April 7, 2000
Accepted May 18, 2000)